How do ants self-medicate?

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Jason Rissanen

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Tiivistelmä – Referat – Abstract

Ants are among the most successful organisms in the world. They can be found almost anywhere on the planet and due to their high degree of sociality and complex societies they have become some of the most abundant creatures in most terrestrial ecosystems. Although sociality has benefits in the form of more efficient foraging, brood care, reproduction and protection from predators, it has costs too. Ants live in high densities in their nests and have frequent contact between them which can facilitate an efficient transmission of pathogens within the nest.

Ants have become highly successful in spite of their potentially high susceptibility to pathogens. They share the same innate immune responses of other arthropods and have unique adaptations for coping with pathogens. In extension to physiological strategies for coping with pathogens, ants engage in behavioural strategies as well. Ants and other eusocial insects can also harness the structure and behaviour of the colony to prevent and cope with pathogen infections through social immunity. Ants can also engage in self-medication behaviour to combat disease. Self-medication is a behavioural strategy where individuals respond to pathogen infections by seeking out and using biologically active compounds to alleviate the effects of pathogens in a way that would be detrimental for uninfected individuals. The behaviour can be either therapeutic of prophylactic depending on when the compounds are used in relation to encountering the pathogen, and it can be extended beyond the self to other kin.

While ants have been proven to medicate themselves with reactive oxygen species (ROS) in laboratory conditions, it remains unknown how they do it in the wild. In my thesis, I studied how the ant *Lasius platythorax* self-medicate in a natural setting by developing a multi-trophic system of ant – pathogen – aphid – plant interactions. In this system, the ants infected with a fungal pathogen (*Beauveria bassiana*) had the opportunity to forage on the nectar produced by the extrafloral nectaries (EFNs) of a broad bean plant (*Vicia faba*) infested by vetch aphids (*Megoura viciae*). Plants that are stressed by aphids react with a systemic production of ROS, which ants are known to use for self-medication, and ROS could therefore be present in the EFN nectar as well, along with other potentially medicinal compounds. The aphids themselves could present the ants with both ROS, if it accumulates in the aphids due to the immune responses of the plant, and protein if eaten.

In my thesis I found out that infected ants increase their foraging on EFN nectar during the first three days after infection compared to healthy ants. This immediate response to a pathogen infection shown by the infected ants fits in a self-medication context as well as the infection cycle of the pathogen, making this a strong case for self-medication. The change in foraging by the infected ants did not reflect on the changes in ROS content in the ants, possibly due to a lack of ROS in the nectar, but instead were likely to be caused by self-generation of ROS in the infected ants. The aphids feeding on the plant contained a higher ROS content compared to the ants, but I found no evidence of ants preying on the aphids, possibly due to the *M. viciae* being unpalatable for the ants or the ants finding medicinal compounds in the EFN nectar.

The result of my thesis is a first step in to identifying natural ways for ants to obtain and use medicinal compounds from their environments and opens up new avenues of research in the topic of self-medication. The result also highlights the importance of biodiversity for the conservation efforts for ants and other insects. Insects are facing a drastic decline in both abundance and diversity due to human impact on their environments, including the prevalence in pathogens. By understanding the full extent of the immune strategies that insects use, including self-medication, we can develop more efficient methods of conservation to help them.

Avainsanat – Nyckelord – Keywords
self-medication, ants, Lasius platythorax, Beauveria bassiana, multi-trophic interactions, insect immunity, behaviour

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Tiivistelmä – Referat – Abstract

Myror hör till de mest framgångsrika organismerna i världen. Deras höga grad av socialitet och komplexa samhällen har hjälpt dem att sprida sig till de flesta terrestriska ekosystemen runt världen. Fastän socialitet har sina positiva sidor, det kan leda till mer effektiv furagering, reproduktion, yngelvård och försvar från predation, så har det sina negativa sidor också. Myror lever i kompakta kolonier och har konstanta interaktioner mellan individer, som kan underlätta spridningen av patogener i kolonin.

Myror har blivit framgångsrika trots deras potentiellt höga risk för sjukdomstransmission. Myror delar samma immunresponser med andra leddjur och har unika adaptationer i kampen mot patogener. Förutom fysiologiska strategier i kampen mot patogener, så använder sig myror av olika strategier av beteende också. Myror och andra eusociala insekter kan använda sig av kolonins struktur och beteende för att förhindra sjukdomar från att sprida sig i kolonin genom social immunitet. Myror kan också använda sig av självmedicinering mot hotet av sjukdomar. Självmedicinering är ett beteende i vilket individer söker och använder sig av biologiskt aktiva föreningar i respons av en infektion för att bota sig mot symptomen på ett sätt som vore skadligt för friska individer. Självmedicinering kan vara antingen terapeutisk eller profylaktisk beroende på när medicineringen sker i relation till när individen har utsatts till patogenen. Självmedicinering kan också riktas till andra släktingar av individer.

Myror har tidigare bevisats kunna medicinera sig mot en svampsjukdom med hjälp av reaktiva syreradikaler (RS) i laboratorium omständigheter, men det är ännu oklart hur de skulle göra det i naturen. I min Pro gradu -avhandling studerade jag hur *Lasius platythorax* myror självmedicinerar i naturliga omständigheter genom att utveckla ett multitrofiskt system var myror, patogener, bladlöss och växter har interaktioner med varandra. I mitt system hade myror som var infekterade med en patogen (*Beauveria bassiana*) en möjlighet att söka föda från extraflorala nektarier (EFN) på bondbönor (*Vicia faba*) som var infesterade med bladlöss (*Megoura viciae*). Växter som är under stress av bladlöss reagerar med en systemisk respons av RS produktion, vilka myror kan använda sig av i självmedicinering. För att RS produktionen är systemisk, så är det möjligt att EFN nektarn också innehåller RS samt andra föreningar med medicinsk potential. Bladlössen kan också fungera som en källa för RS ifall det ackumulerar i bladlössen som en följd av växternas immunrespons, samt som en källa för protein som behövs för att upprätthålla myrornas immunförsvar.

Resultaten från mitt projekt visar, att infekterade myror furagerar mera EFN nektar under de tre första dagarna efter att blivit insjuknade jämfört med friska myror. Denna klara respons till infektionen och infektionscykeln av *B. bassiana* tyder på aktiv självmedicinerings beteende. Furageringen av EFN nektar reflekterade inte RS nivåerna i myrorna, vilket kan bero på att EFN nektarn inte innehåller RS, men istället var ökningen av RS i infekterade myror på grund av självproduktion. Bladlössen innehåll en klart högre nivå av RS, men jag hittade inga bevis på att myrorna skulle ha ätit bladlöss under experimentet, möjligtvis på grund av att M. *viciae* är oätbar för myror eller för att myrorna hittade vad de behövde för självmedicinering från EFN nektarn.

Resultaten i min Pro gradu -avhandling är ett första steg för att identifiera naturliga sätt hur myror hittar och använder sig av biologiskt aktiva föreningar från deras omgivning för självmedicinering. Detta resultat lyfter fram hur viktig biodiversiteten är för skyddsåtgärder för myror och andra insekter. Insekternas mängd och diversitet har sjunkit drastiskt under de senaste åren på grund av människans påverkan på deras omgivning, inklusive på prevalensen av patogener. Genom att förstå hur insekter använder sig av immunstrategier som självmedicinering, kan vi utveckla bättre sätt för att skydda dem.

Avainsanat – Nyckelord – Keywords
Självmedicinering, myror, Lasius platythorax, Beauveria bassiana, multitrofiska interaktioner, insekt immunförsvar, beteende

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Table of contents

1. Introduction

Insects are the most diverse terrestrial animals on earth making up roughly two thirds of all terrestrial species. Being ubiquitous, many ecosystems rely on the services that insects provide such as pollination, seed dispersal, decomposition and regulating population sizes (Folgograit 1998; Rico-Gray & Oliveira 2007; Schowalter et al. 2018). An alarming decline in insect populations around the world has been recorded in recent years, causing concern for the future of many ecosystems. According to recent literature, up to 40% of all insect species may be facing extinction in the coming decades (Sánchez-Bayo & Wyckhuys 2019). The most prominent reasons that are affecting the decline of insects are either caused or accelerated by human activity through, for example, the destruction of habitat, intensive agriculture, the use of pesticides, pollution, pathogens, and climate change (Sanchez-Bayo & Wyckhuys 2019). Whereas human impact on insect populations has been relatively brief and insects are still catching up to adapt to it, insects have been interacting with pathogens for a long time and have developed many ways to cope with them.

1.1 Insect immune systems

Insects have an innate immune system which helps them fight against the pathogens they face. The innate immune system of insects comprises centrally of a physical barrier, the cuticle, as well as chemical defenses against pathogens.

The cuticle is a chitinous outer shell of the insect exoskeleton which functions as a physical barrier, separating the inside and outside environments of all insects (Vincent & Wegst 2004). The cuticle is largely impenetrable for most pathogens, with mostly fungi being able to penetrate it directly with the help of enzymes that the spores secrete (Siva-Jothy et al. 2005). The cuticle does have some weak spots at the joints, which can present the pathogens with possible entry points to reach the inside of the insects.

Insects have cellular and chemical mechanisms to contain and kill the pathogens that successfully breach the cuticle. There are hemocytes in the insect hemolymph that have phagocytic activity to encapsulate and kill pathogens that they encounter (Lavine & Strand

2002). Insects can also synthesize a wide variety of antimicrobial peptides able to lyse the membranes of the pathogenic cells (reviewed in Yi et al. 2014). An essential part of insect innate immunity are enzyme cascades. Both the intermediate forms and the end products of the cascades can be used in the fight against pathogens. Melanin, which is used to encapsulate and isolate pathogens inside the insect in a process called melanisation, is produced by the phenoloxidase cascade, which also plays a part in the production of reactive oxygen species (ROS) (Söderhäll & Cerenius 1998). ROS are comprised of highly reactive and volatile oxygen molecules, formed through the reduction of oxygen, that play a central part in insect immune signaling (Mikonranta et al. 2014). Apart from its role in signaling, ROS can also be used to kill pathogens directly as it can cause tissue and genetic damage due to oxidative stress caused by its high reactivity, but a prolonged state of high oxidative stress is also harmful for the host (Ryu et al. 2006).

Insects were thought for a long time to lack the ability to mount an increasingly efficient immune response to repeated exposures of the same pathogen similar to the adaptive immunity known in vertebrates. This was due to a perceived lack of immune cells capable of storing the pathogen response as well as the relatively short lifespan of insects. However, there has been mounting recent evidence of insects being capable of priming their immune systems to repeated exposures of the same pathogen (reviewed in Contreras-Garduño et al. 2016). There is also evidence of insects priming the immunity of their offspring against a specific pathogen that their maternal parent has faced (Sadd et al. 2005).

1.2 How do ants cope with pathogens?

Ants are among the most successful organisms in the world. They can be found almost anywhere on the planet and due to their high degree of sociality they have become some of the most abundant creatures in most terrestrial ecosystems (Bronstein 2001; Hölldobler & Wilson 2009). A colony of ants can contain millions of individuals that can forage in a wide area around their nest, leading to interactions with a wide array of other species in their surroundings. Being numerous and capable of aggressively dominating animals much bigger than themselves, ants are often a sought-after partner in mutualistic interactions where the partners exchange services for a net benefit for both (Bronstein 2001). Ants often provide

protection for mutualistic species in exchange for nutrients, but also provide services in seed dispersal, pollination and fertilization (Rico-Gray & Oliveira 2007). Being opportunistic omnivores, ants can also control the populations of other species through predation. Through their interactions with other species as well as the effects on the soil they often build their nests in, ants can have a great influence on the ecosystem they reside in. In many cases, ants have a disproportionally high effect on the shape, function and condition of ecosystems and are therefore often referred to as ecosystem engineers (Folgograit 1998). Although ants are still numerous around the world, the same afflictions that are wreaking havoc among other insects are affecting ants as well. Human caused changes in land management and pesticide use along with pathogens use are causing a decline in both density and diversity of ant species around the world (Folgarait 1998).

1.2.1 Immune system and special adaptations

Sociality has been key in the rise of the ants in the world and it has several benefits. It allows for more efficient foraging, brood care, reproduction and protection from predators (Bourke 2011). However, sociality has its costs too. A high density of individuals and frequent contact between them can facilitate an efficient transmission of pathogens within a social group (McCallum 2001). Most ants also share food with one another through trophallaxis, the sharing of regurgitated food to another ant, which is also potentially an effective way for a pathogen to spread within a colony (Schmid-Hempel 1998).

Ants have become highly successful in spite of their potentially high susceptibility to pathogens and parasites (Schmid-Hempel 1998). Ants share the same physiological immune responses with other insects and arthropods. They use melanisation to encapsulate pathogens and they have hemocytes in the hemolymph to combat with pathogens on a cellular level. Ants are also able to synthesize a variety of antimicrobial peptides. There is also evidence of immune priming in ants (Fuchs et al. 2018) although it is still scarce, and some species of ants seem to lack the ability to prime their offspring against certain pathogens (Galvez & Chapuisat 2014).

In addition to the innate immune system that ants have in common with other insects, ants also have special adaptations unique to them that help them in their constant combat with

disease. Ants have multiple glands that produce antibacterial and antifungal compounds that they use to process nest material brought into the nest as well as applying it topically on themselves to prevent pathogen intake from the environment (Rico-Gray & Oliveira 2007; Hölldobler & Wilson 2009). The secretions of the metapleural glands in ants are antimicrobial and function against several different pathogens when spread on the cuticle (Veal et al. 1992). The exudates of the poison glands of some ant species are shown to also prevent pathogen growth (Tragust et al. 2013; Brütsch et al. 2017). Apart from relying on their own physiological adaptations, leaf-cutter ants are also known to have mutualistic bacteria growing on their cuticle that protects the ants from entomopathogenic fungi (Mattoso et al. 2011).

1.2.2 Social immunity

The social structure of eusocial insects that can facilitate efficient pathogen transmission can be used to prevent it as well. The workers in a colony are assigned to different tasks and group themselves accordingly. These groups have limited interactions between each other which can limit the spread of a pathogen in the colony (Mooring & Hart 1992; Naug & Smith 2007; Cremer et al. 2018). The outside foragers of the colony are most at risk for pathogen exposure, and the individuals of the group of outside workers often interact mostly with other outside workers (Hölldobler & Wilson 2009). The inside workers mostly keep themselves inside the colony, away from the pathogen rich outside environment, and are primarily working on brood care and nest maintenance.

Individual ants in a colony display collective behaviours that together can reduce the effect of a pathogen in the colony in the form of social immunity. Social immunity is a term coined by Cremer et al. (2007) and is defined as the altruistic behaviours of social insect individuals that reduce the colony's overall pathogen load and pressure. Individual behaviours include the honest display of infection on the cuticle of sick individuals which triggers avoidance, caring or aggressive behaviours of nestmates toward the infected individual (Schmid-Hempel 1998). On a colony level, the social immunity strategies often consist of behaviours for the avoidance, resistance and the tolerance of pathogens (Cremer et al. 2018).

Avoidance of pathogens is often the first step in preventing pathogen spread within a colony, where the individuals avoid areas or foods rich in pathogens to reduce the risk of pathogen intake (Tranter et al. 2015). Avoidance behaviours also include the avoidance of infected individuals as well as the social exclusion of infected individuals, denying them entrance to the nest (Drum & Rothenbuhler 1983; Bos et al. 2012). Avoidance is not entirely clear at times, as there are also contradictory reports of ants being attracted to places that are rich in pathogens (Pontieri et al. 2014).

Resistance includes all behavioural responses that actively reduce the overall pathogen load in the colony. The pathogen load of an ant colony can be effectively controlled through different hygiene and sanitary behaviours. Hygiene maintenance is an integral part of keeping the pathogen load low inside the ant nest as clearing out debris and waste effectively reduces possible spots for pathogen proliferation (Hart et al. 2002). Maintaining a high level of individual hygiene as well as taking care of your nestmates hygiene, a behaviour called allogrooming, is common for ants and can also help in keeping pathogen load and risk of transmission low in the colony (Theis et al. 2015). In addition to keeping the colony and its members clean and hygienic, it can also be essential to keep organic materials brought into the colony clean and pathogen free. Leaf-cutter ants use the anti-fungal exudates of the metapleural glands to disinfect the organic matter that they bring into the colony to keep bacteria from infecting the fungus that the ants cultivate for food (Fernandez-Marin 2006).

Tolerance includes the behaviours that help the ants to cope with the detrimental effects and symptoms of the pathogen without necessarily affecting the overall pathogen load in the entire colony (Soares et al. 2017). Tolerance is still poorly studied and understood but is becoming an increasingly interesting topic for further research (Cremer et al. 2007; Ayres & Schneider 2012). It is suggested that understanding the role of self-medication against pathogens might give insight to understanding how tolerance works (de Roode & Hunter 2019).

1.2.3 Self-medication in ants

Self-medication is the deliberate use of medicinal compounds to fight or prevent a pathogen infection (Parker 2011; Abbott 2014). The compounds are sought out from the environment and used through ingestion, absorption, topical application or proximity (Clayton & Wolfe 1993). For something to be considered true self-medication, it has to fulfill four criteria: (1) the substance must be deliberately sought out and contacted, (2) the use of the substance increases an infected individual's fitness, (3) the amount of the substance used by an infected individual is harmful for an uninfected individual, (4) the substance must be harmful for the pathogen (Clayton & Wolfe 1993; Singer et al. 2009; Abbott 2014). Recently there has been more discussion over the criteria for self-medication. De Roode et al. (2013) questioned the need for the substance to be harmful for the pathogen for a behaviour to be classified as self-medication, arguing that if an animal uses a compound to better tolerate the negative effect of the pathogen without affecting the actual fitness of the pathogen, it should be considered as a self-medicating behaviour as well.

Self-medication can be either prophylactic or therapeutic and it can be directed at either on the individual level or to genetic kin and offspring (Figure 1) (Lozano 1998; de Roode & Hunter 2019). The fact that the target of self-medication behaviour can extend beyond the self to kin has caused discussion about whether it would be more accurate to talk about animal medication instead of self-medication (de Roode & Hunter 2019). In this thesis I will continue with using the term self-medication for medicating behaviour which is directed both at the self or kin. The self-medication behavioural response can be both qualitative, where the individuals are incorporating the use of new compounds in their behaviour, or qualitative, where the individuals increase the use of compounds that are a part of their natural environment to compensate for the loss or need of that compound to combat a pathogen (Lozano 1998).

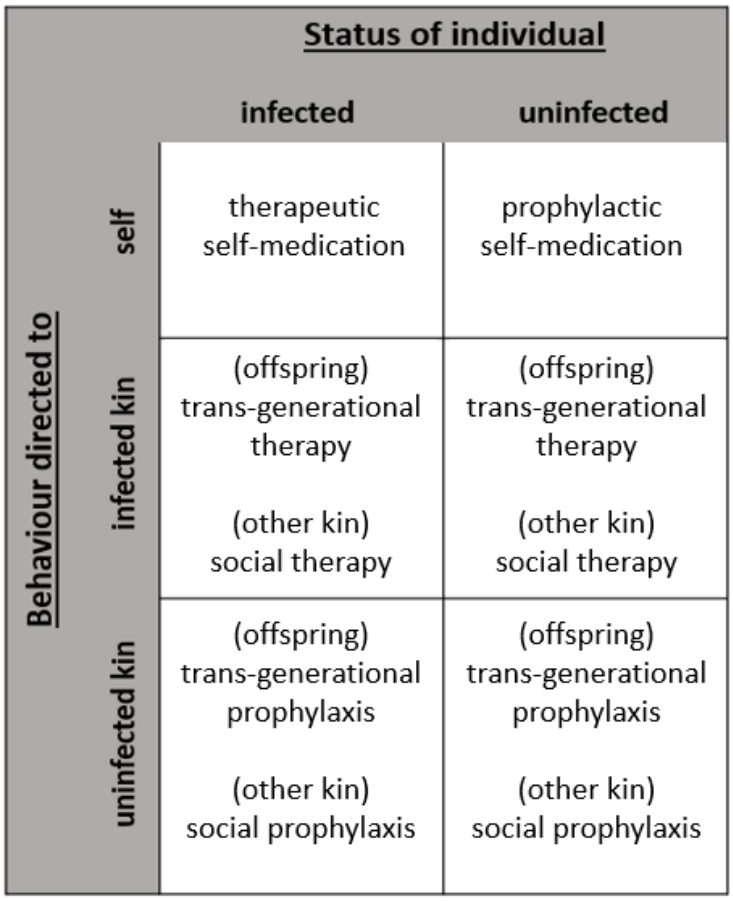

Figure 1 Self-medication explained with the status of the individual displaying the behaviour and to whom the behaviour is directed at. Self-medication behaviour can be directed beyond the individual self to both infected and uninfected kin and can occur both prior and during infection. Picture modified from de Roode & Hunter (2019).

Prophylactic self-medication is the use of medicinal compounds prior to the infection taking place, responding to a heightened risk of infection, which raises the fitness of individuals when the infection takes place or lowers the risk of infection. Prophylactic behaviours includes the collecting and storing of products with anti-microbial properties. Many plant-produced compounds, such as resin, have anti-microbial properties and insects like bees and ants are known to forage for resin and incorporate it into their nest material to help keep pathogens in check inside of the nest (Simone-Finström & Spivak 2012; Brütsch 2017). Wood ants further process the collected resin with formic acid to increase the ability of the resin to inhibit pathogen growth within the nest (Brütsch et al. 2017). The response to increase the amount of resin into the nest is caused by the presence of brood instead of infection, making the behaviour prophylactic rather than therapeutic.

Therapeutic self-medication is the use of medicinal compounds when the infection has taken place. Due to its complex nature it was long thought to be a trait of only higher vertebrates. However, evidence of therapeutic self-medication in insects is increasing (Lee et al. 2005; Milan et al. 2012; De Roode 2013; Abbott 2014; Bos et al 2015). Caterpillars of *Spodoptera littoralis* reacted to an infection with an increase in protein intake in a study by Lee et al. (2005) to successfully combat a pathogen infection. Protein is important for the upkeep of immune responses in insects, and the amount of protein that the larvae ingested was on a level that was shown to be harmful for healthy larvae, proving a case for a quantitative response of self-medication. *Drosophila* larvae have been reported to exhibit a qualitative self-medication response (Milan et al. 2012). When parasitized by a wasp, the *Drosophila* larvae changed their feeding preference from ethanol-free food to food that contains ethanol to lower the parasite fitness. The parasitized larvae that changed their feeding preference to food which contained ethanol had higher survival compared to larvae that were feeding on an ethanol-free diet. However, when uninfected, the larvae who fed on an ethanol containing diet had a lower fitness compared to larvae feeding on an ethanol-free diet.

Ants have been shown to successfully engage in therapeutic self-medication behaviour in laboratory settings. *Formica fusca* workers used a diet enriched with ROS to successfully alleviate the detrimental effects of an infection by the entomopathogenic fungus *Beauveria bassiana* (Bos et al. 2015). In the experiment, ants who were infected foraged more on food that was treated with a ROS (H_2O_2). When presented with foods treated with different concentrations of ROS, the infected ants chose to forage on the food with a specific concentration of ROS to obtain a dose which is lethal for the pathogen but not too harmful for themselves. This choice of the right dose implies that the ants were not randomly choosing their food but showed intent to identify and use a correct amount of ROS to treat the infection. This change in foraging behaviour caused by the infection led to a higher survival of infected ants compared to infected ants that had no access to ROS treated food.

Prophylactic self-medication is widely discussed in the context of social immunity as it can increase the ability to cope with pathogens on a colony-level, but therapeutic self-medication has rarely been mentioned in the same circumstances. There is now evidence of therapeutic self-medication in social insects such as ants and bees (Gherman et al. 2014; Bos

et al. 2015) where the workers can medicate themselves when infected, but it was discussed more on the level of individuals rather than at the colony-level although the potential for it is evident in both cases. While trophallaxis in ants is considered to be a behavior that increases pathogen transmission risk, ants can share their individual immune responses to nestmates during trophallaxis, thus increasing pathogen tolerance (Hamilton et al. 2011). If ants who forage on ROS containing food for the purpose of self-medication share it with its nestmates through trophallaxis then this could be considered an altruistic behavior as the individual ant is sharing valuable medicinal compounds with a nestmate at a possible cost to itself to raise the fitness of another. If ants do share the medicinal compounds obtained from foraging, then therapeutic self-medication should be considered as a part of the social immunity framework.

1.3 Sources of ROS for ants in nature

While there is speculation of how ants would self-medicate in nature, there is no evidence yet on where or how they obtain compounds such as ROS for self-medicating in the wild and whether it works in the same way as it does in laboratory conditions. Sapolsky (1994) raised the argument of the need for self-medication experiments to be studied in more natural conditions. Many of the experiments are concentrating on isolated single compounds (Singer et al. 2009; Bos et al. 2015) but these compounds might behave differently when isolated compared to their natural state in the source.

Ants can generate ROS in their bodies in response to an infection as it is a component in the enzyme cascades in the innate immunity of insects (Söderhäll & Cerenius 1998; Ryu et al. 2006) or obtain it through their diet as shown in the experiment by Bos et al. (2015). Ants are often opportunistic foragers and they face possible sources of ROS during foraging. Decaying organic matter such as carcasses have a high concentration of ROS (Pasckowski & Schütz 2011) and ants are known to forage on carcasses in the wild.

As is the case with insects, ROS plays a central part in the immune responses of plants both directly and as secondary messengers in the immune signaling pathways (Cruz de Carvalho 2008). Plants that are stressed by herbivore damage such as an aphid infestation display a systemic response of elevated ROS and its precursors to combat the herbivores damaging

the plant and to protect the wounded areas from pathogens (Cruz de Carvalho 2008; Duran-Flores & Heil 2014). As the reaction of elevated ROS is systemic, there is reason to believe that nectar produced by plants would be a plausible source of ROS in a plant that is suffering of a herbivore infestation.

Plants produce nectar to attract and reward mutualistic partners such as insects who act as pollinators and as protection from herbivores. Plants can have nectaries both in the floral structure (floral nectaries) (Figure 2) or outside of it (extrafloral nectaries, EFNs) (Figure 3). Floral nectar is mainly for the attraction of pollinators but is known to contain ROS to prevent bacterial contamination of the nectar (Escalante-Pérez et al. 2012). The nectar produced by the EFNs is mainly for the attraction for insects such as ants for protection against herbivores (Rico-Gray & Oliveira 2007). Nectar contains energy in the form of carbohydrates and is central in the diet for adult ants and the colony spends significant effort to forage for nectar (Hölldobler & Wilson 2009). The nectar produced by floral nectaries and EFNs in some plants contain different sugars (Davis et al. 1988), so the quality of the nectar varies depending on the need for specific carbohydrates and can attract different insects to the different nectaries. Apart from carbohydrates, nectar also contains a variety of other compounds including alkaloids and amino acids in smaller amounts (Nepi et al. 2014). Although the contents of the nectar produced by the EFNs are not well known, there are signs that it contains anti-fungal agents to prevent contaminations of the nectar (Escalante-Pérez et al. 2012). The secretion rate of nectar from the EFNs is also highly correlated to ROS formation in the plant caused by herbivore damage (Duran-Flores & Heil 2014).

Figure 2 Floral nectaries on a broad bean plant. The floral nectaries are hidden inside of the floral structure and are commonly associated with pollinator rewards.

Figure 3 Extrafloral nectaries on a broad bean plant. The EFNs are outside of the floral structure on the petioles of the plant and are often affiliated with rewarding symbiotic insects for protection against herbivores. The EFNs of the broad bean are often highlighted by black dots on the plant (circled with blue).

Aphids are homopteran herbivores that suck the phloem of plants and are common pests of many agriculturally important plants, causing damage and functioning as disease vectors (Baryanovits 1973). An infestation of aphids on a plant is capable of inducing a defensive ROS response in plants which has a detrimental effect on the aphids (Bi & Felton 1995; Bednarski et al. 2013). Some species of ants have mutualistic interactions with several species of aphids, where the aphids reward the protection provided by the ants with the excess sugar of the phloem, a secretion known as honeydew (Offenberg 2001; Hölldobler & Wilson 2009). Even if the relationship between ants and aphids was mutualistic, the ants are still known to occasionally prey on the aphids (Sakata 1994). If the plant is combatting the aphids with a ROS response, it is possible that the honeydew contains ROS that infected ants could use to medicate themselves with. However, as ants readily collect honeydew it is likely that the honeydew does not contain high levels of ROS. If ROS accumulates in the aphids as a result of the defensive response of the plant, it could explain the detrimental effects on the aphids (Bi & Felton 1995), the aphids themselves may present ants with a source both ROS and protein needed to combat disease (Lee et al. 2005; Bos et al. 2015).

1.4 The experiment and hypotheses

My aim in this thesis was to try to identify a natural way for ants to use ROS for self-medication. I observed the foraging behaviour of colonies of the ant *Lasius platythorax*, and whether the foraging behaviour will differ depending on whether the colony suffers from an infection or not. *L. platythorax* is a very common species of ants in the palearctic ecozone and was described as its own species by Seifert (1991), separated from its sibling species *Lasius niger* which has been readily used in studies on foraging dynamics on aphids and nectar (Katayama & Suzuki 2004). *L. platythorax* shares a lot of morphological similarities to *L. niger* but differ for example in their choice of habitat. *L. platythorax* is more common in woodland areas and often uses rotten wood as nest material, whereas *L. niger* prefer open areas and often nests in soil. *L. platythorax* is an opportunistic forager and often preys on invertebrates as well as tends aphids for honeydew and forages on other sources of sugar such as EFNs.

I infected half of the colonies with *B.bassiana* for the experiment, and the rest were left as sham treated control colonies. The ant colonies foraged on broad bean plants (*Vicia faba*) infested with vetch aphids (*Megoura viciae*). The broad bean is an annual plant that develops EFNs on its stipules and the ants had access to the nectar produced by them. Broad bean plants are known to use ROS as a systemic response to herbivore damage (Ederli et al. 2017), and therefore the nectar that they produce may contain ROS for the ants to use. The ants also had the option of directly using the aphids as food. Aphids contain protein, which is normally lacking in nectar, that insects also need for their immune defence (Lee et al. 2005). The vetch aphid is a species of aphid that uses leguminous plants including the broad bean as a host. They do not engage in mutualistic behaviours with ants, such as presenting honeydew (Novgorodova 2002). There is no direct evidence of ants preying on vetch aphids (Novgorodova 2002) which are considered to be generally unpalatable and potentially toxic to some aphid predators (Tsaganou et al. 2004). If the plant is upregulating ROS to combat the aphid infestation as has been shown, it is also probable that the aphids have a high ROS content, and therefore could be more attractive for ants that are combating a pathogen infection.

Our experimental setup gave the infected ants a choice of a quantitative response on increasing foraging on the nectar produced by the plant or a qualitative response on supplementing on aphids. The experiment is able to fulfil the criterions to identify self-medication behaviour. We know from Bos et al. (2015), that ants can seek out and dose themselves with the right amount of ROS to combat the disease. As higher exposures to ROS can be harmful for healthy ants but can help infected ants to kill the pathogen, raising their own fitness while lowering the fitness of the pathogen, our set up covers the criteria for self-medication: 1 (the substance must be deliberately sought out and contacted), 2 (the use of a substance increases an infected individual's fitness), 3 (the amount of the substance used by an infected individual is harmful for an uninfected individual) and 4 (the substance must be harmful for the pathogen).

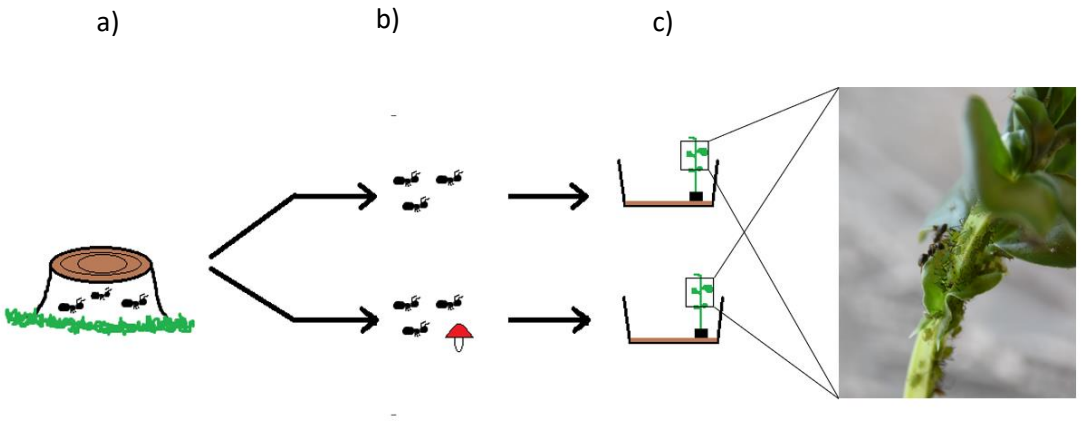

Figure 2 Scheme of the experimental setup. Workers of L. platythorax *were collected from natural nests (a). The workers from each nest were split into two sub-colonies of which one was infected with the entomopathogenic fungus* B. bassiana *and the other functioned as a healthy control (b). The colonies were placed in individual identical nest-boxes which contained a broad bean plant infested with vetch aphids (c). The ants in both treatments did therefore have equal opportunity to either forage on the EFNs located in the base of the stipules or the aphids on the plant.*

My hypotheses are:

(1) The ants in the infected colonies will forage on the nectaries more actively.

If the infestation of aphids causes an elevated ROS response in the plants, the nectar that they produce will be more attractive to the ants in the infected colonies.

(2) Infected ants will eat aphids.

Aphids are a source of protein needed for the upkeep of an immune response against infection and are also a possible source of ROS if it accumulates in them due to the response of the plant against herbivore infestations.

(3) The infected colonies will have a higher ROS content in accordance to a higher foraging on nectar or aphids.

If the nectar or aphids contains ROS, it should accumulate more in the ants that forage more on them.

2. Material & Methods

2.1 Experimental setup

The experiment was conducted at the Tvärminne zoological station in June 2019 in Hanko, Finland. I collected workers and nest material from 20 colonies of *L. platythorax* ants from a wood clearing in Lappohja, Hanko, Finland (59°54'46.9"N, 23°15'53.8"E, Figure 5) into plastic buckets and stored in a cold-room overnight in +4°C in wait for further processing.

Figure 3 The wood clearing where the nests of L. platythorax *were collected from on the left picture. The* L. platythorax*, pictured on the right, often nests in rotting wood such as old tree stumps.*

I counted 1000 ants from each nest and separated them into two duplicate sub-colonies for a total of 40 colonies containing 500 workers each. No queens were collected into the experimental colonies. The colonies were placed into plastic nest-boxes (35 × 20 cm and 20 cm high) lined with a mixture of ethanol and talcum powder (20% v/v) which has been considered to be effective in preventing ants from escaping (Ning et al. 2019). The nest-boxes contained a 2cm layer of gardening peat and a ceramic tile (10 × 10 cm) to serve as nest-material.

The aphids were sent to us from the Julius Kühn Institute for Plant Protection in Germany by Dr. Torsten Will on broad bean stems (Figure 6). I raised the aphids on stock plants of broad bean and allowed them to freely reproduce to achieve sufficient numbers for the

experiment. I counted and transferred 27 ± 3 aphids for each plant chosen for the experiment. The variance in the number was an attempt to account for the difference in the size of the individual aphids.

*Figure 4 Vetch aphids (*Megoura viciae*) on a broad bean (*Vicia fabae*) plant*

The plants chosen for the experiment were not flowering but had developed at least one pair of EFNs. Each plant was planted separately in a small plastic pot (6 × 6 cm and 6 cm high) with a wooden skewer to support the plant. The aphids were given time over night to infest the bean plant before introducing the pots to the ant nest-boxes.

2.2 Infections

One of the pair of duplicates from each original nest was infected with spores of the generalist entomopathogenic fungus *B. bassiana*, which is commonly found in Finnish soil. To prevent any effect of local adaptations, I used a Danish strain (KLV 03-90) which has been successfully used with *Formica fusca* (Bos et. al 2015).

In order to confirm that the fungus can also be used with *L. platythorax*, I collected spores from multiple plated fungi to create a solution of suspended spores of *B. bassiana* in Milli-Q water to be used for the infections. To quantify the concentration of spores in the solution, I counted the number of spores under a microscope by using a haemacytometer. I then diluted the solution to reach the desired concentration of 1 × 10^7 spores/mL for the infection trials. To test whether the fungus was still infective and lethal to *L. platythorax*, I set up an infection trial of four duplicates of four colonies of *L. platythorax* in small jars (20 ants/jar). Two of the duplicates of each colony were infected with *B. bassiana* and the other two served as controls. The colonies were then observed for mortalities for a total of seven days and provided with fresh food (Bhatkar & Whitcomb 1970) and water every day.

The ants that were assigned for the infections were collected into a specially made dipper constructed from a cut-off Falcon tube and mesh (Figure 7). The dipper containing the ants was then dipped into a container containing the spore solution for five seconds. The ants were then dried fast with an absorbent paper towel then returned into their respective jars. The spore solution was mixed between each suspension by shaking the jar containing the solution. The ants assigned to the control treatment were dipped in a container containing Milli-Q water for five seconds. Once confirmed that the fungus was capable of infecting and killing *L. platythorax*, I proceeded with the infections for the experiment.

The infections for the experiment were conducted using the same protocol, however I decided to use a concentration of 1 × 10^8 spores/mL to make sure that the ants got infected with the fungus. The ants were then given time overnight to settle after the procedure before the observations started.

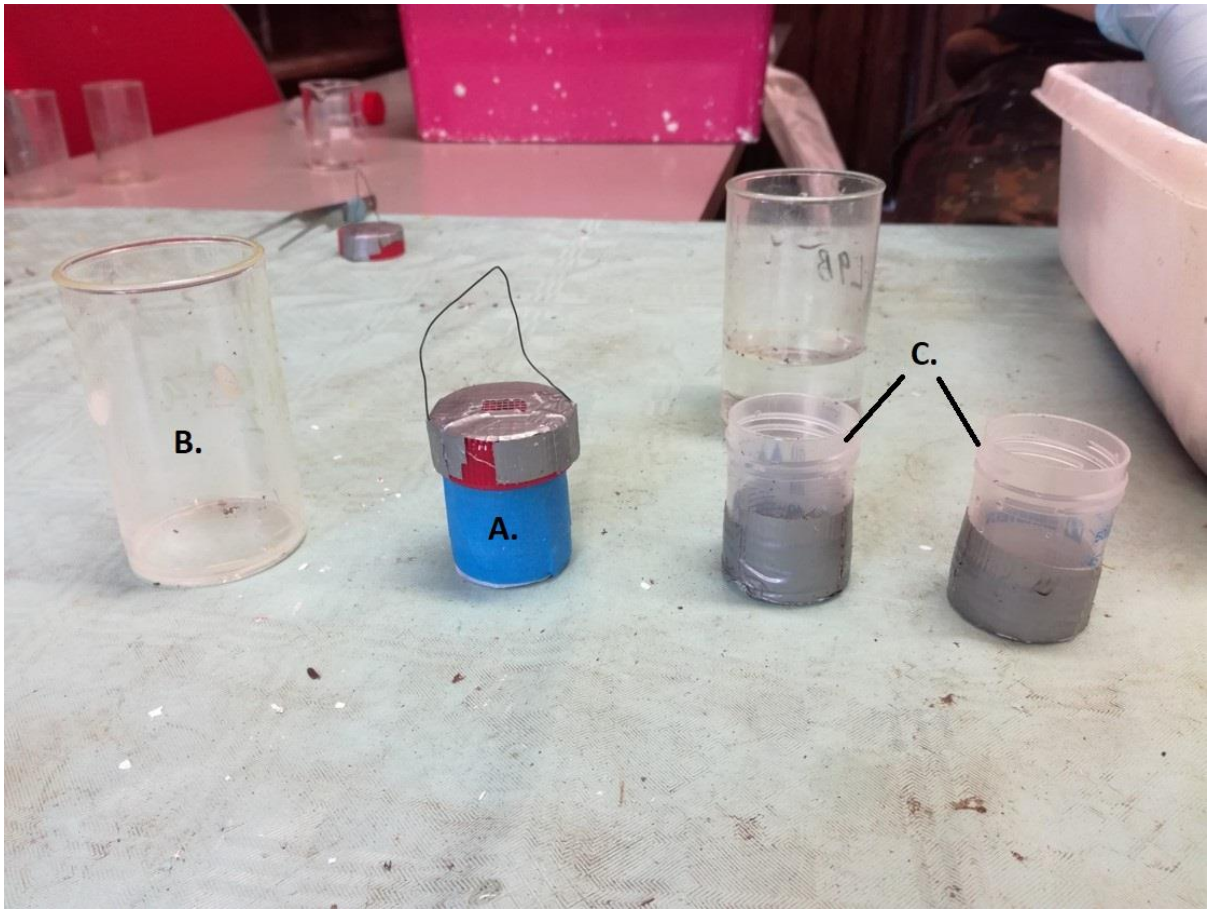

Figure 7 The dipper (A.) was constructed by cutting a 50mL Falcon tube in half. The bottom of the tube was covered in mesh to prevent the ants from falling out. Once all the ants were collected into the dipper, the dipper was submerged into a container (B.) which would hold the spore or control solution, and stay submerged for 5 seconds. For the control treatments I used different dippers (C.) to prevent any spore contamination to the controls.

2.3 Observations

During the observation period, I counted the number of ants foraging on the EFNs in each colony. An ant facing the EFNs on the stipules while being within a body length of them was classified as a forager on the EFNs (Figure 8). I also made note on contact on any aphids by ants. The point scale for scoring the aphid interactions was a scale from 0 – 2. If there was no purposeful contact the observation was scored as a 0. If the ants showed interest in the aphids with antennation or licking, the observation was scored as a 1. If the ants behaved aggressively towards the aphids by pulling on legs, biting or carrying the aphids towards the colony, the observation was scored as a 2. The foraging behaviour of the ants was observed six times per day: 9 a.m., 11 a.m., 1 p.m., 3 p.m., 5 p.m. and 7 p.m. for a total of six days.

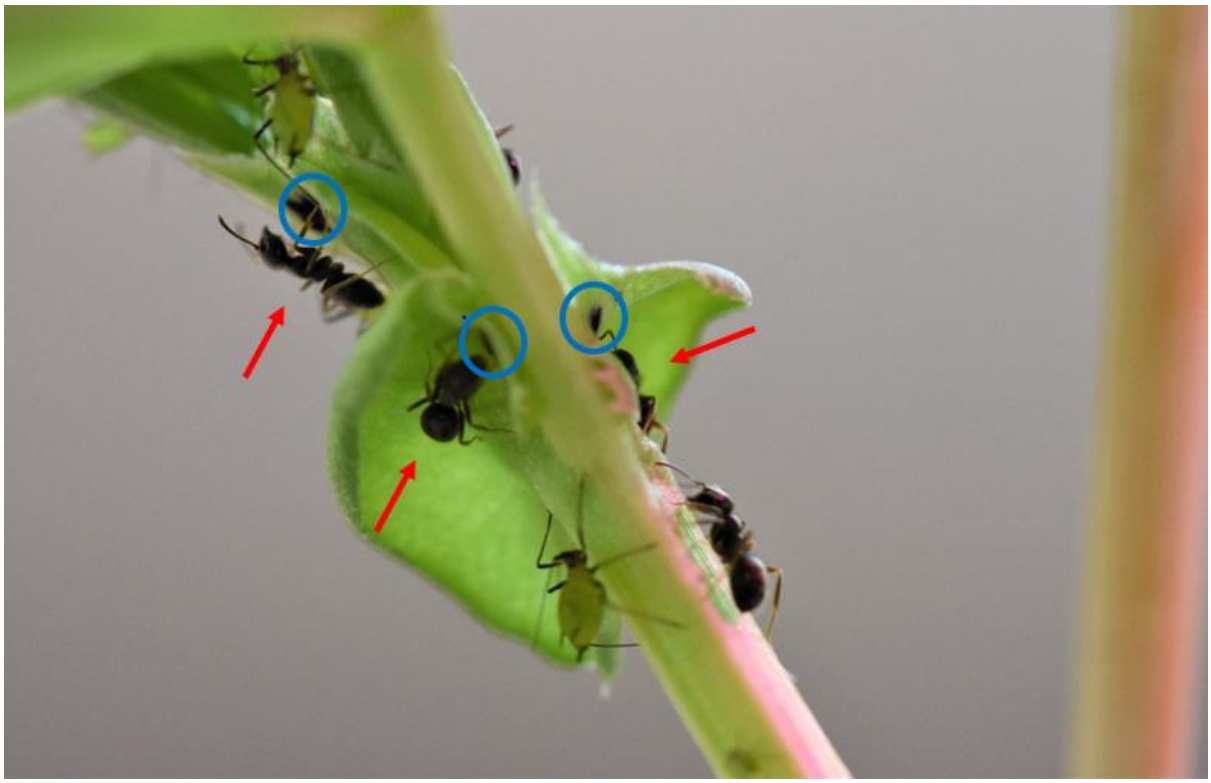

Figure 8 The three ants that are marked with red arrows are considered foragers on the extrafloral nectaries, which are circled with blue. The ants are facing the nectaries and they are all within a body length of them.

The aphid infested plants were introduced to the nest boxes on day 1 at 9 a.m., and the first observations were made at 7 p.m. on the same day to give the ants time to get acquainted with the plant. The nest-boxes were placed randomly in the room to try to negate the effects of placement in consideration to the door and windows, and the order of the boxes was shuffled every two days. I placed two plant lights (Airam plant LED E27 5W) to provide additional light to the colonies residing further away from the windows in an attempt to ensure the wellness of the plants. The plants were watered every day at 3 p.m. after observing the foraging behaviour. To prevent observer bias I implemented a blinding procedure. The colony information was not present on the nest-boxes. Each box was assigned a number from 1 – 40 by another person, so that the observer would not know which colony resides in which nest-box and which treatment the ants in each box has had. The colony information was sealed and revealed only after the experiment was terminated.

I used two video cameras to try to capture evidence of ants using aphids as food. The cameras were set to focus on the base of the plant in the nest-box. This way, if an ant carries an aphid to the nest, it will have to pass through the frame of the camera. The colonies were filmed for 30 minutes at a time at 9 a.m., 10 a.m., 11 a.m., 12 p.m., 1 p.m., 2 p.m., 3 p.m., 4 p.m., 5 p.m. and 6 p.m. The schedule for the filming was done by the same person who assigned the final coded numbers for the nest-boxes to ensure that both infected and healthy colonies were filmed in equal amounts.

Figure 9 The experimental set-up. All 40 nest-boxes were placed on two counters. The counter further away from the window was additionally lit with two plant lights to ensure that the plants got enough light to stay healthy. The boxes were also shuffled randomly every 2 days to negate any effects caused by the position in the room.

2.4 ROS analysis

I sampled ants and aphids for ROS analysis to see whether the levels of ROS vary between the species, timepoints and treatments, and if this could explain any difference in foraging dynamics. Ants were sampled in the beginning of the experiment (day 0), the middle (day 3)

and on the morning of day 7 after the experiment had been terminated. Aphid samples were taken on day 7. Three biological replicates (10 ants, 15 aphids/sample) were taken on each sampling event and placed in separate Eppendorf tubes with 250µl 1 × PBS 3-amino-1,2,4-triazole solution (2mg/mL) to prevent the ROS from reacting. The samples were then stored immediately in -80^{o}C.

The ants chosen for sampling were taken randomly from the nest-boxes. Both foragers on the plants and individuals found underneath the ceramic tile were chosen to find evidence that the entire colony would benefit from the collected ROS through sharing of food by trophallaxis within the colony, and not just the primary foragers.

The analysis of ROS content in the samples was done at the University of Graz in Austria in January 2020. The samples were homogenized by placing two steel marbles into each Eppendorf tube containing an ant or aphid sample and then shaken in a TissueLyser II (Qiagen) for five minutes at 1800 rpm. The homogenized samples were then centrifuged, and the supernatant was collected from the centrifuged samples for the analysis of both protein and ROS contents.

The protein content was analyzed using the Bicinchonic Acid Protein Assay Kit (Sigma-Aldrich) according to the protocol provided by the manufacturer with minor adjustments, by using 12,5µl of sample in 100µl of working reagent, keeping the same 1:1 stoichiometry as mentioned in the protocol. I ran a test first to see what concentration of sample with the reagent would provide results that fit in the standard curve. I ended up using a dilution of 2,5µl of ant samples in 10µl of 1 × PBS. For the aphid samples, I used 5µl of the aphid sample in 7,5µl of 1 × PBS. The absorbance of each sample was read on 562 nm wavelength in a microplate reader (SpectraMax iD3, Molecular Devices).

The ROS content was analysed with the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen). The analysis was done according to the protocol provided by the kit manufacturer with minor adjustments, by using 25µl of sample in 25µl of reagent, keeping the same 1:1 stoichiometry as mentioned in the protocol. I ran a test to see what concentration of sample used would give us results that fit in the standard curve. I ended up using a dilution of 12,5µl of ant samples in 12,5µl of reagent buffer. For the aphid samples I used 5µl of aphid sample in 20µl of reagent buffer. The dilutions were corrected for in the

final calculations. The fluorescence of the samples was read on 573 nm – 608 nm after excitation in 530 nm – 560 nm in a microplate reader (SpectraMax iD3, Molecular Devices). I calculated the ROS/protein content of the samples to avoid differences in the sizes of ants or aphids that could affect the results.

2.5 Statistical analysis

All statistical analyses were performed using the R software (R Core Team 2019, https://www.R-project.org/, version 3.6.2). All the graphs were built using the *ggplot2*-package (Wickham 2016).

I used the *survival*-package (Therneau 2020) to analyze the results of the initial infectivity test of the fungus. I used mortality events as a response variable in the model with treatment as the explanatory variable.

For the analysis of the foraging data, I used the number of foragers as the response variable and the treatment as the explanatory variable. I used the original nest as a random factor in the analysis since the original nests were split into two sub-colonies for the experiment to be used in the separate treatments, to account for the non-independence of sub-colonies originating from the same nests. The plants in two of the nest-boxes of control colonies died during the experiment and these colonies were therefore omitted from the statistical analyses.

I used the *glmmTMB*-package (Brooks et al. 2017) which allowed me to build regression models with random effects to analyze the foraging data. In the experiment by Bos et al. (2015), the change in foraging behaviour was immediate upon infection by a pathogen, so the foraging data was analyzed for both the first three days as well as the full six-day period. I fitted poisson- and negative binomial models to the data and calculated the Akaike's information criterion (AIC) and the Bayesian information criterion (BIC) for the models, which is considered to be a robust way to find the best model to fit to the data (Pho et al. 2019). In the data for both the 3-day and 6-day data, the negative binomial model was a better fit compared to the poisson model.

I used the *lme4*-package (Bates et al. 2015) for analyzing the results of the ROS contents of the samples. I used the ROS/protein content as the response variable in the model with treatment as the explanatory variable and the initial nest as a random factor in the model.

3. Results

3.1 Infections

The fungus was successful in infecting and killing workers of *L. platythorax* (N=280, df=1, X^2=20, p<0.0001). 97.5% of all the ants who received the control treatment were still alive seven days after the treatment, whereas only 61.5% of the infected ants survived during that time. Most of the mortalities in the infected colonies occurred after day 4 (Figure 10).

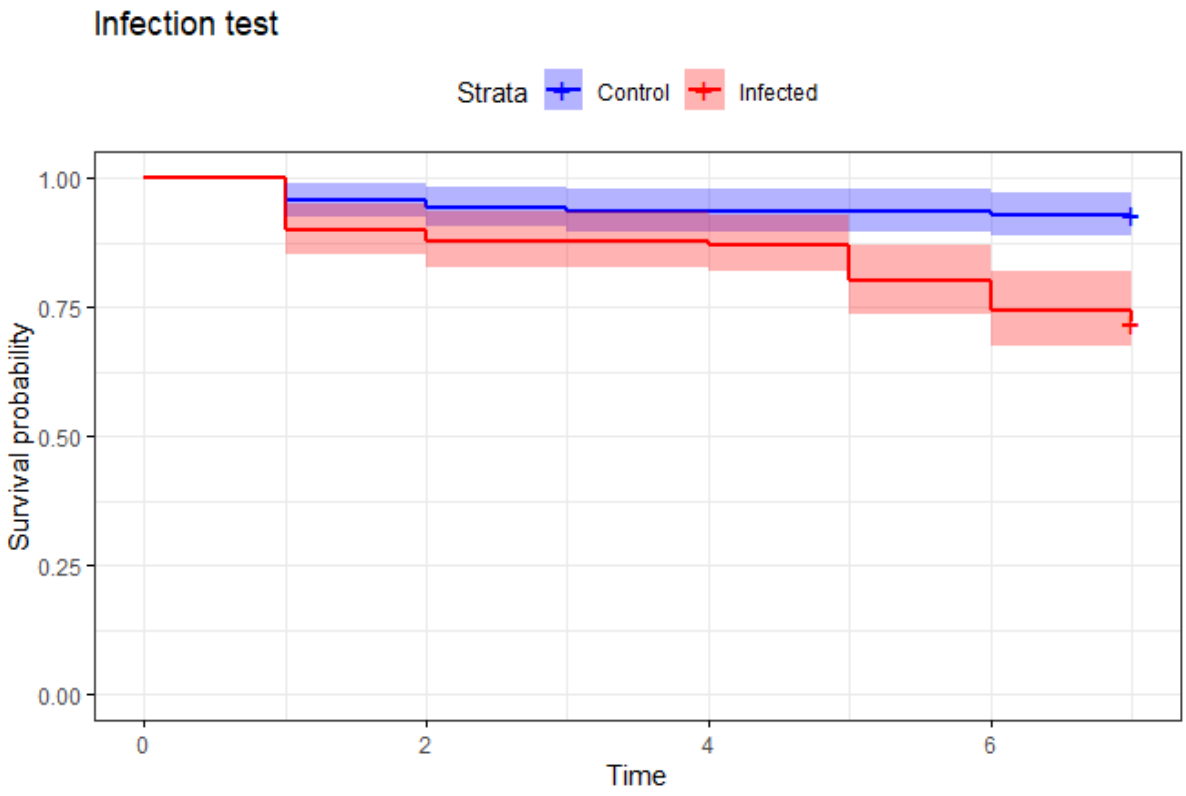

Figure 10 Survival graph of the infection test. The y-axis represents the probability of survival and the x-axis denotes time in days. B. bassiana *was successful in infecting and killing workers of* L. platythorax *(N=280, df=1, X^2=20, p<0.0001). Most of the mortalities occurred after day 4. The red line indicates the survival of the infected colonies and the blue line represents the control colonies. The shaded area along the lines indicates the 95% confidence interval.*

3.2 Foraging

During the first three days, the workers of the infected colonies were foraging more on the nectaries compared to the healthy colonies by a factor of 1.23 (N=684, z=2.11, p=0.035, Figure 11a). After the full six days, no difference in foraging could be detected (N=1368, z=-0.56, p=0.58, Figure 11b).

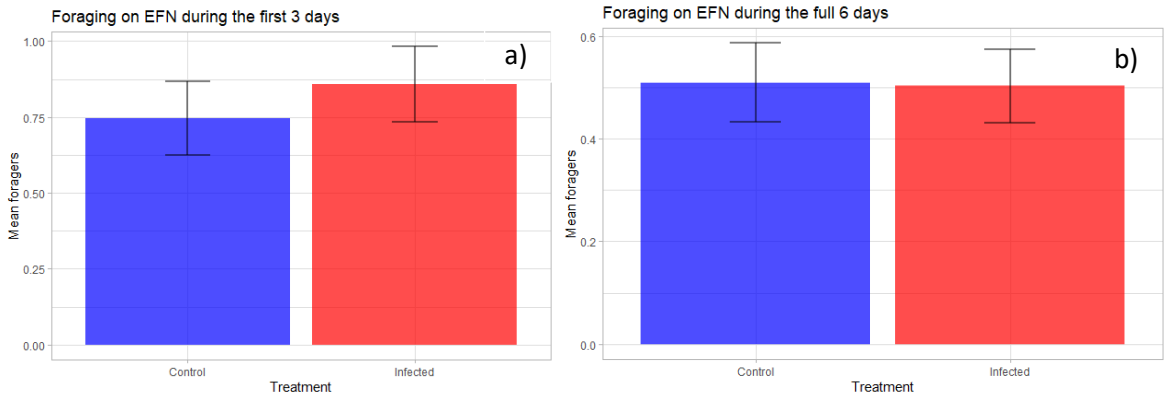

Figure 11 The foraging activity during the first three days (a) and the full 6-day observation period (b). The y-axis denotes the mean number of foragers found foraging at the EFNs at any time. The infected colonies (red) were foraging more compared to the control colonies (blue) during the first 3 days (N=684, z=2.11, p=0.035) (a). No difference in foraging was detected over the whole 6-day observation period (N=1368, z=0.56, p=0.58) (b). The error bars represent the 95% confidence interval.

There was no difference in contact with the aphids between the control and infected colonies (N=684, z=0.027, p=0.98). One of the video cameras used to record the colonies for evidence of ants using aphids as food suffered a software malfunction, causing the loss of almost half of the overall footage. The remaining footage of 62 separate recording events, 31 hours of footage in total, was spread out evenly between infected and control colonies. The remaining footage showed only one instance where ants were acting aggressively towards the aphids. A worker from an infected colony picked up one aphid from the plant and then carried to the nest, but the ant quickly returned with the aphid and dropped discarded it away from the colony.

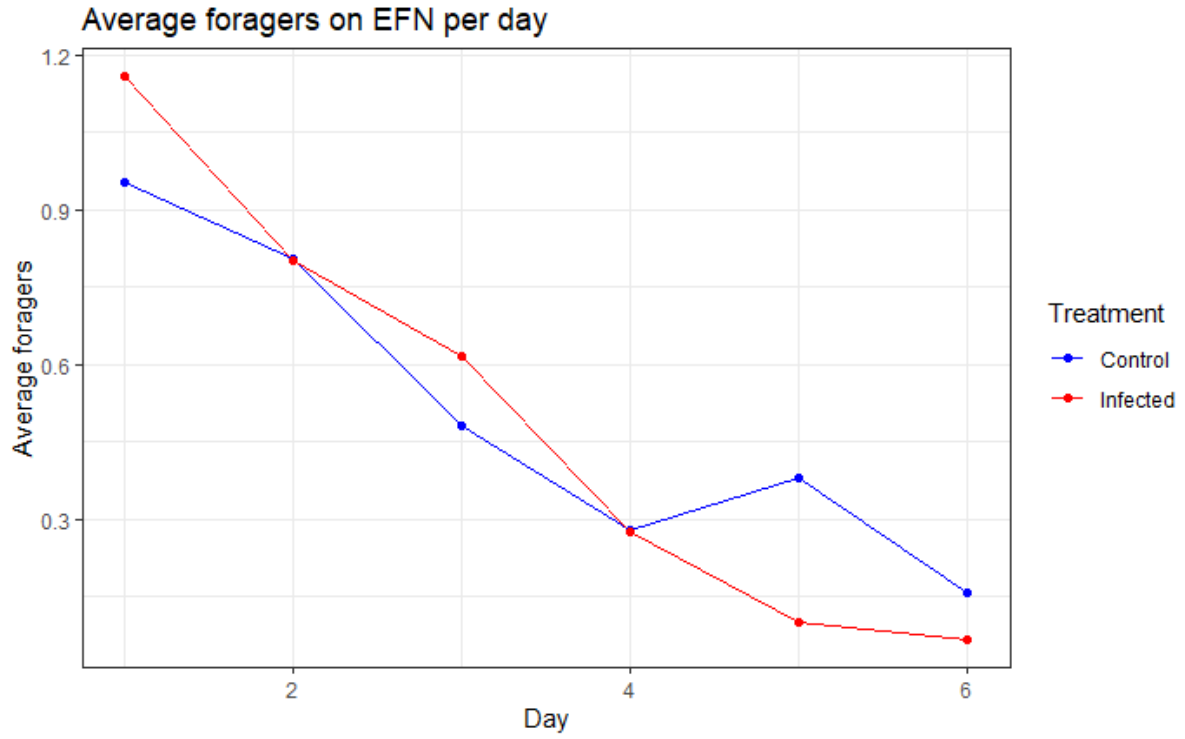

Figure 12 Foraging dynamics in time. The infected colonies (red line) had more foragers on average compared to the control colonies (blue) during days 1 and 3. The average amount of foragers is the same during days 2 and 4, and the control colonies have more foragers on EFNs during the last two days. The overall trend of foraging on EFNs is decreasing in time in both the infected and control colonies with the exception of day 5, where the control colonies show a slight upturn in foraging before decreasing again.

3.3 ROS analysis

The infected colonies showed a higher ROS/protein content than the control colonies in the 7th day samples (N=114, df=95, t=2.55, p=0.012, Figure 13). No significant difference in ROS/protein content was found during the first three days (N=114, df=95, t=-0.44 p=0.66). Aphids contained a clearly higher content of ROS/protein compared to the ants on day 7 (N=226, t=-25.24, df=206, p<0.0001). There was no significant difference in the ROS/protein content of the aphids in the different treatments (t=-1.33, df=97, p=0.19).

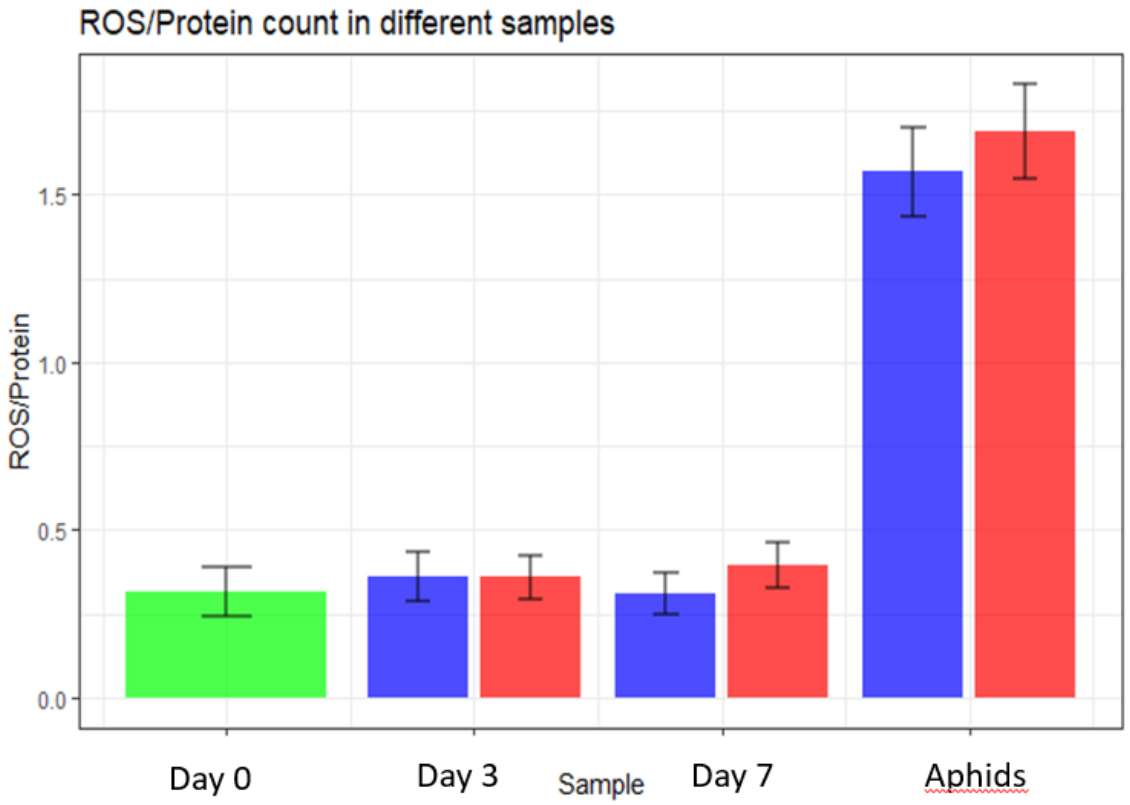

Figure 13 The differences in ROS/protein content in mg/mL in each sample. Day 0 is marked with green because the samples were taken prior to any infection procedures. In the day 3, 7 and aphid samples, the control colonies are marked with blue and the infected colonies are marked with red. The ants of the infected colonies had a significantly higher ROS/protein content in the day 7 samples compared to the control colonies (N=114, df=95, t=2.55, p=0.012). The aphid samples contained a significantly higher ROS/protein content compared to the ants on day 7 (N=226, t = -25.24, df = 206, p < 0.0001). The error bars represent the 95% confidence interval.

The infected colonies also showed an increase of ROS/protein content in time (N=180, df=159, t=2.36, p=0.02), but no increase was detected in the healthy colonies (N=168, df=150, t=0.20, p=0.84, Figure 14).

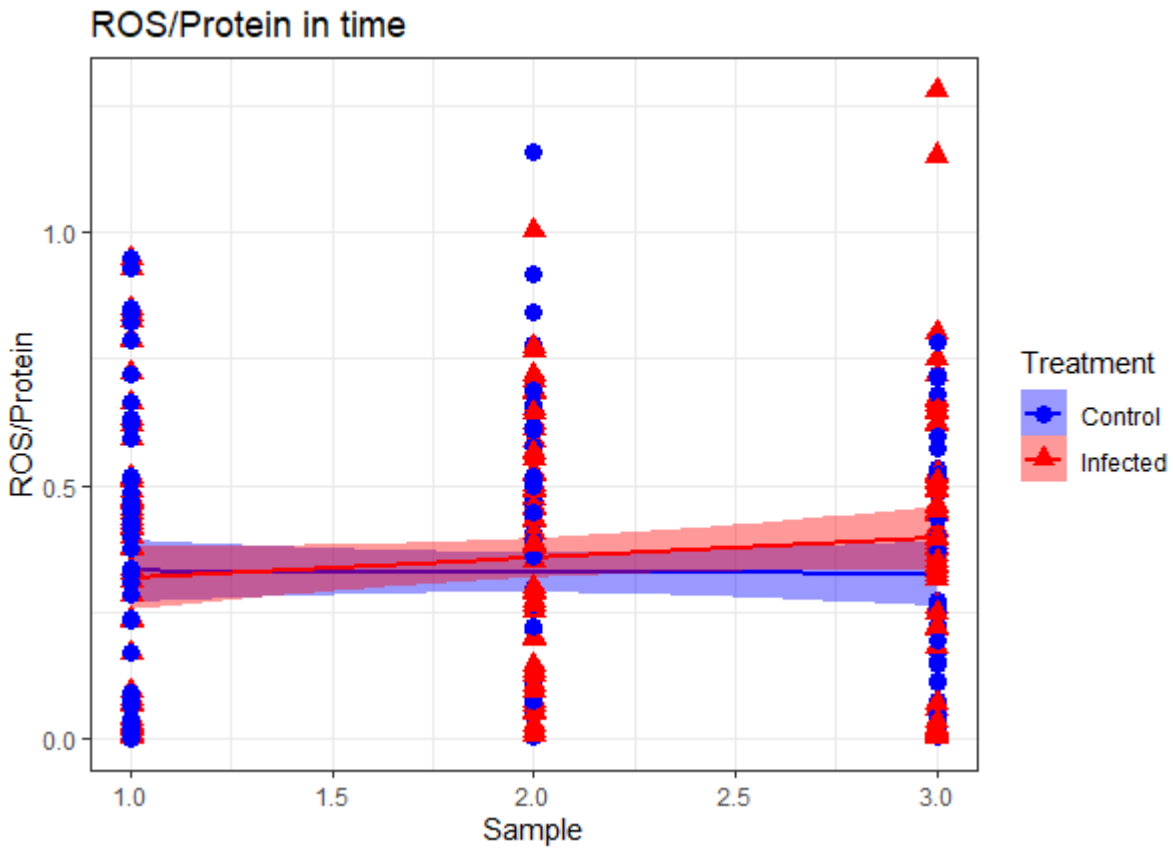

Figure 14 The changes in ROS/protein contents in mg/mL in time denoted by samples. The ROS/protein content increases in the infected (red) colonies from day 0 to day 7 (N=180, df=159, t=2.36, p=0.02). No increase was detected in the control (blue) colonies (N=168, df=150, t=0.20, p=0.84). The shaded area on the lines represent the 95% confidence interval.

4. Discussion

In this experiment I have shown that an infection by the entomopathogenic fungus *B. bassiana* elicits an immediate response in *L. platythorax* to increase the frequency of foraging on nectar produced by the EFNs of the broad bean plant. The experiment provided no evidence of ants changing their behaviour towards *M. viciae* aphids or incorporating them into their diet in response to an infection by *B.bassiana*. We did find that the infected ants had an increase in ROS in time, whereas the healthy ants not exposed to the fungus had no change in their ROS content. There was no clear evidence of foraging significantly affecting the ROS level in the ants.

4.1 Pathogen induced change in nectar foraging

The observations I made during the first three days fits my first hypothesis: that the ants in the infected colonies forage more actively on the nectaries compared to the control colonies. This represents a quantitative change in foraging behaviour in response to a pathogen infection, which is in accordance with the criteria for therapeutic self-medication. Other self-medication studies on insects have shown the same immediate response to exposure to a pathogen which we observed in our experiment (Bos et al. 2015). The overall foraging during the full six days shows no difference between the two treatments. When looking at the foraging dynamics of ants on EFNs in time (Figure 12), it seems that the healthy colonies overtake the infected colonies in foraging during the last two days of the experiment.

There could be many reasons why the number of workers foraging on the nectaries falls to a lower level compared to the healthy colonies in time. If the attempted self-medication by the infected colonies was successful because of the increase in foraging during the first three days, the colonies could compensate on feeding less immediately afterwards to alleviate detrimental effects caused by the use of biologically active compounds. According to the third criterion of self-medication, the substance used for self-medication is detrimental for healthy individuals if used in the same amount (Abbott 2014). Therefore, if the ants have obtained medicinal compounds in such an amount which is detrimental for

them once the infection has been has been cleared, they could limit the intake of food containing these compounds until they reach an internal level which is tolerable for healthy individuals. However, if the attempted self-medication by the infected colonies was unsuccessful in preventing the pathogen from killing the ants, the overall number of ants in the infected colonies would be lower compared to the ones in the healthy colonies. In the infection test conducted to verify that the pathogen was successful in infecting and killing the ants, we started to see infected ants die in greater numbers after four days. If the ants in the infected colonies were to suffer more deaths during the last days of the experiment, it could explain why the overall number of foragers in the infected colonies drop.

The actual mortality in the colonies could not be counted due to a few cases of ants escaping from their nest-boxes. I do, however, believe that even though there were escapees in a few of the colonies, it would not significantly affect the foraging results, which were robust enough for us to believe that infection, not escapees, was the cause of the detected change in foraging on the nectaries.

4.2 Foraging on aphids

I did not observe a qualitative change in diet, i.e. the use of aphids as a food source, caused by infection in the ants as I predicted in my second hypothesis. There was no observable difference in type of contact between control or infected ants and aphids either. The video footage shows only a few cases of aggressive contact and one case of carrying an aphid in the observations and video footage, but nothing concrete to suggest the use of aphids as supplement in their diet. The vetch aphid is considered to be potentially toxic and generally unpalatable (Tsaganou et al. 2004) and it could be that this cost outweighs the ants need for protein or ROS, which the aphids did contain to a much higher degree compared to the ants (Figure 13). It is also possible that the ants found what they were looking for in the nectar and therefore the use of aphids as food was not needed.

The video footage did show that ants of both infected and control colonies spent time licking or chewing the skewer that was meant to provide support for the plant. As these are commercially produced skewers, there is a possibility that they were coated with something that the ants considered attractive. Due to the fact that the licking of the skewers was

something both infected and control colonies engaged in, it is unlikely that it has anything with self-medication, but it is a factor I need to take into consideration for future experiments.

4.3 Changes in ROS content

The ants in the infected colonies show an increase in ROS content in time, whereas the ants in the healthy colonies showed no change in ROS content throughout the experiment. The increase in ROS content in the infected colonies was not steep, and a difference in ROS could not be detected between the infected and healthy colonies during the first three days. The increase in ROS does not reflect the foraging dynamics, therefore does not comply with my third hypothesis.

Because the changes in ROS content in the ants did not reflect their foraging dynamics, it is possible that the increase in ROS was due to self-generation (Söderhäll & Cerenius 1998) instead of successfully obtaining it through their diet. The infected colonies were foraging more actively on the nectar compared to the healthy colonies during the first three days of the experiment, yet no difference in ROS content between the two treatments could be detected at that time. After six days, no difference in foraging could be detected between the two treatments, but at that time the infected ants contained a significantly higher amount of ROS compared to the ants in the control colonies. The control colonies foraged continuously on the nectar produced by the plants under herbivore stress yet showed no changes in ROS content throughout the experiment.

There is a possibility that we have missed possible differences in ROS levels caused by foraging due to the way we sampled workers for the ROS analysis. We sampled ants randomly from the nest-boxes. If the ants did not share the nectar throughout the colony, there is a chance that differences in ROS levels in individual ants would not show. If the primary foragers that collected nectar in the colonies were to contain a higher level of ROS but not share it, then our method of sampling random ants could hide increases of ROS in the primary foragers. This would be true for both the infected and control colonies. To correct for this possible sampling error, we could build replicate samples of mostly foragers,

non-foragers and a mixture of both to see if there is an increasing gradient of ROS contents from the non-foragers to the primary foragers.

4.4 Are aphid stressed plants a source of medicine for ants?

The lack of a change in ROS content in the healthy colonies despite continuous and cumulative foraging of nectar could be due to a lack of ROS in the nectar of the EFNs, unlike what I assumed. This could mean that although the ROS production in the plant is systemic, there are mechanisms in place to prevent the ROS from being present in the EFN nectar (Davis et al. 1988). This could provide stronger evidence for the role of EFNs in protection against herbivores through predator attraction (Engel et al. 2001; Katayama & Suzuki 2004; Rico-Gray & Oliveira 2007). If the nectar produced by EFNs would contain elevated amounts of ROS in response of herbivore damage, then the nectar would be less attractive due to the presence of harmful compounds. There is evidence of ants switching their foraging preference from nectar to honeydew on a broad bean plant if an infestation of a species of aphids which engages in mutualistic interactions with ants grows larger (Katayama & Suzuki 2003). This was accredited to the fact that there is a higher volume of honeydew produced compared to nectar, but it would be interesting to test the nectar for elevated levels of ROS and whether that could be an influence the switch in resource preference.

If the extrafloral nectar were to be ROS free, it still contains a multitude of different compounds, and there is continued research for identifying its composition (Nepi 2014). Nectar produced by EFNs contains a high concentration of secondary metabolites, such as non-protein amino acids and alkaloids, which are known to affect the behavior of insects including ants (Kessler & Baldwin 2007; Wright et al. 2013; Cammaerts et al. 2014; Nepi 2014; Grasso 2015). Some alkaloids found in floral nectar, such as nicotine, has also been linked with medicinal use by bumblebees (Baracchi et al. 2015). Some of the most common non-protein amino acid found in nectar produced by EFNs are GABA (gamma-aminobutyric acid), beta-alanine and taurine (Nepi 2014). All three of them are common in the nervous systems of animals, where they regulate the excitability of neurons. GABA has also been proven to affect the behaviour of insects by stimulating the pace of food intake (Nepi 2014). The ants of the infected colonies could have been attracted to other compounds present in

the nectar and identifying the composition of the nectar could be the key to uncovering what causes the attraction. The increase in nectar foraging could also be due to a higher energy demand when combating an infection, and not a need for a particular compound in the nectar.

A factor which I could not control in the experiment was the variation among plants. The plants had differences in environments even though I tried to minimize the effect of it. The aphids reproduced more effectively on some plants than on others as well as the fact that some plants had the availability to direct sunlight might cause differences in the physiology, including the ROS metabolism, of the plant, potentially affecting the quality and contents of the nectar (Grasso 2015; Sun et al. 2020). If the nectar of some plants would be more enriched with ROS or other compounds, then the ants foraging on that plant could forage differently on it compared to ants with access to other plants. To take the individuality of the plant into consideration, I would have to take nectar samples of each plant at specific times. This way it would be possible to correct the foraging activity with the concentrations of compounds produced by the plant in real time, because it is known that ants can dose themselves with just the right amount of ROS to fight fungal pathogens (Bos et al. 2015).

In a natural environment, ants would have access to both floral and EFNs. The composition of the nectar produced by the different nectaries on a plant is different, with floral nectar known to contain ROS in some plants (Escalante-Pérez et al. 2012), plants could therefore present the ants with a source of ROS even though it would not be present in the nectar produced by the EFNs. In the experiment, I focused on the foraging on nectar produced by the EFNs as it makes up for a substantial part of the diet of ants (Rico-Gray & Oliveira 2007). Although the nectar of the EFNs seemed to lack ROS, a plant that suffers from an aphid infestation could still be a source of ROS for ants through the floral nectar or aphids, but further research is needed to confirm this.

Even though the aphids contained a higher amount of ROS compared to the ants, I could not find evidence of ants using them as food. The aphids would have been a source of protein and ROS for the ants and both have been proven to be used in a self-medication context (Lee et al. 2005; Bos et al. 2015). The aphid species we used for the experiment is not known to be preyed upon by ants, but ants are known to prey upon other species of aphids which could provide the ants with at least the protein that they need to medicate themselves

against a pathogen. Whether they contain a sufficient amount of ROS to be used by ants for medicating would still need to be investigated.

4.5 Future outlook

Identifying the natural sources that insects use to obtain medicinal compounds is important for the conservation efforts against the recent alarming population declines of insects around the world. While there is mounting evidence of insects being capable of self-medicating (Abbott 2014; de Roode 2019), many of these studies are focused on single isolated compounds instead of a more natural environment (Singer et al. 2009; Bos et al. 2015). In my thesis, I tried to identify a natural way for ants to obtain and use biologically active compounds to medicate themselves against an entomopathogenic fungus which is rich in their natural environments by foraging on a broad bean plant that is suffering from an aphid infestation. In this system, the ants could take advantage of the stress reactions raised by the plant resulting in elevated ROS levels to fight against the aphids by foraging on the nectar produced by the stressed plant or to forage on the aphids that contain protein needed to maintain an immune response.

Although the foraging dynamics did not point to ROS being present in the EFN nectar, ROS was still present in the aphids in the system. The vetch aphids is not a species of aphids that is affiliated with ants and is considered unpalatable (Novgorodova 2002; Tsaganou 2004), but they could be substituted for a species of aphids which is palatable in future experiments to see whether ants would supplement their diet with aphids that are edible. The saliva of aphids can affect the physiology of plants, including their immune responses, differently according to their compatibility to the host plant (Mondal 2020; Sun et al. 2020), so experimenting with different species of aphids could alter the composition or the concentration of compounds of the nectar as well. My plans for the continuation in this topic of research includes the use of different species of aphids on plants to see, whether the presence of different species of aphids on the plant affects the survival of infected ants differently. The effect of the different aphid species could be due to either the aphids affecting the plant differently or the ants being able to feed on different aphids. Further studies of this matter could also aim for plants with both floral- and extrafloral nectaries, as

floral nectar reportedly contains ROS in some plants (Escalante-Pérez et al. 2012). This way it could be possible to see whether there would be differences in foraging ratios between floral and EFN nectar between infected and uninfected ants, and if those differences would reflect on the survival of infected ants.

Analysis of the nectar composition of aphid infested plants is going to be a crucial factor which needs to be addressed in future use of this system for studies of self-medication research. Nectar composition analysis would provide more insight into the identification of compounds which have medicinal properties that ants would be able to use upon infection. infected ants were initially foraging more on the EFN nectar in the presence of vetch aphids. Comparing the nectar composition in the presence of different species of aphids would also show how the different species of aphids affect the biochemistry of the plant.

Although it has been proven that ants can self-medicate themselves, the question over whether they share the medicinal compounds they obtain to their nestmates as a form of social therapy remains. Methods for the analysis of the regurgitate that ants share with each other through trophallaxis have been developed (Hamilton 2011), and they could be harnessed to investigate whether ants would share medicinal compounds such as ROS to other infected ants as well. There are also methods for the tracking of the dissemination of food by ants in a colony (Greenwald et al. 2015) which could be used to follow whether the food shared by primary foragers has positive effects on the survival of other infected nestmates downstream in the interaction network, which would also be a sign of social therapy.

5. Conclusions

The results of the experiment confirm my first hypothesis, that infected ants initially increase their foraging on EFN nectar on a broad bean plant suffering from an aphid infestation. My experiment could however not confirm my second hypothesis of the infected colonies supplementing their diet with aphids. The change in foraging activity on the nectar did not correlate with an elevated level in ROS in the ants as I predicted in my third hypothesis. The rise in ROS levels of the ants could instead have been due to self-generation of ROS as part of the innate immune reactions of ants.

The results of the experiment also highlight the reason for conservation of natural undisturbed habitats. The way ants and other insects medicate themselves in nature is still unknown, and if we act unknowingly, we might destroy crucial interactions that insects need to procure medicinal compounds to sustain a healthy life. Inter-species interactions have evolved during a long time, and the pace that humans disturb the environment can cause damage to insect populations at a much larger scale than previously imagined if the dynamics of these interactions are affected.

The availability of antimicrobial compounds for insects to use against pathogens is not only affecting the insects but could provide humans with potentially life-saving knowledge. Nature has provided us with several compounds that we use for their antimicrobial and antibiotic properties. By better understanding how insects find, identify and use medicinal compounds as well as fungal and bacterial symbionts to keep harmful pathogens at bay, it may provide us with new medicine against pathogens that are growing resistant to the antibiotics we use today (Ratcliffe et al. 2011). A healthy environment with a large biodiversity is therefore not only crucial for the well-being of insects, but us humans too.

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